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Research Focus

New proteins in the apicoplast membranes: time to rethink apicoplast protein targeting

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Several apicomplexan parasites harbour an essential plastid known as the apicoplast. Apicoplasts import proteins and metabolites for several biological functions, but how import is achieved is largely unknown. Two recent reports have identified novel proteins in the apicoplast membranes, providing new perspectives on how proteins traffic to this organelle. The first report contributes to a newly recognized apicoplast-targeting pathway for membrane proteins, and the second identifies the first member of the protein-translocation complex in apicoplasts.

The apicoplast at a glance

Apicomplexan parasites harbour an apicoplast (apicomplexan plastid), which is a non-photosynthetic homologue of the photosynthetic plastid organelle, or chloroplast, of plants. A pigmented, photosynthetic apicoplast was identified recently in the apicomplexan coral symbiont *Chromera*, suggesting that the apicoplast is degenerate in parasites and, apparently, lost in *Cryptosporidium* [1]. Apicoplasts synthesize fatty acids, isoprene precursors, iron–sulfur clusters and haem [2,3], and these pathways would seem to make the apicoplast indispensable. Interest has focused on unravelling the origin, biogenesis, cell biology and metabolic activities of the apicoplast, to perturb its function with therapeutics and kill parasites. Comparatively little is known about apicoplast membrane proteins; two new papers redress this deficit. DeRocher *et al.* [4] have found a novel redox protein associated with the apicoplast membranes, reminding us of our incomplete understanding of apicoplast metabolism. Van Dooren *et al.* [5] have identified a chloroplast protein import component, Tic20, in the innermost apicoplast membrane, highlighting the retention of ancestral plastid machinery in the apicoplast. Here, we discuss the impact of these findings.

Targeting to the apicoplast without a leader

The apicoplast genomes encode <50 proteins [6], but a large cohort of nuclear-encoded proteins traffic into the apicoplast by targeting peptides on their N termini [7–9]. The targeting sequences are bipartite, comprising a signal peptide (SP), which facilitates the entry of a nascent polypeptide into the secretory pathway, and a transit peptide (TP), which then targets the protein to the apicoplast [8,9]. Detailed characterization of the TP reveals that positive charges are essential for faithful apicoplast targeting [9,10], but how these are recognized is unclear. The SP is removed during co-translational import into the endoplasmic reticulum (ER), and a protease homologous to the stromal processing peptidase (SPP) of plant chloroplasts is proposed to remove the TP within the apicoplast stroma [11].

Genome mining has identified ~500 proteins that are likely to reside in the apicoplast stroma, but little is known about proteins residing in the four membranes that surround the apicoplast (Figure 1). As the gatekeepers between this bacterium-like organelle and the parasite proper, these membrane proteins are potentially excellent drug targets, and their identification and characterization are, thus, important. The first apicoplast membrane proteins identified were two plant-like sugar-phosphate transporters, PfoTPT and PfiTPT, which have been proposed to supply the organelle with carbon, ATP and reducing power [12]. A PfoTPT homologue, TgAPT1, and a protease termed 'TgFtsH1' have subsequently been localized to the outer apicoplast membranes in *Toxoplasma gondii* [13,14]. Recently, DeRocher *et al.* [4] found a novel apicoplast thioredoxin-like protein, ATrx1, that they concluded is associated with the peripheral compartments of the apicoplast (Figure 1). Like other proteins targeted to the outer apicoplast membrane, ATrx1 lacks a canonical bipartite leader, further expanding a new paradigm for apicoplast targeting different to the bipartite leader pathway to the stroma (Figure 2).

Thus far, proteins located in (or adjacent to) the outer apicoplast membrane lack any obvious common targeting motif. Nevertheless, a transmembrane domain seems to act as a signal anchor to commit the polypeptides into the endomembrane system, in which the apicoplast is positioned [8]. To this end, putative signal anchors on TgFtsH1 and ATrx1 were necessary for entry into the secretory pathway [14,4]. From there, it is envisaged that ER-transition vesicles would carry the proteins to the outermost apicoplast membrane (Figure 2). Exactly what anchors TgFtsH1, TgAPT1 and PfoTPT to the outermost apicoplast membrane or subtending compartments is not known (Figure 1). A major difficulty in localizing proteins in the periphery of a multimembrane-bound organelle is resolution. TgAPT1, TgFtsH1 and ATrx1 are thought to reside in more than one apicoplast membrane, and ATrx1 also resides in peripheral subcompartments [13,14,4]. These conclusions are based solely on immunoelectron microscopy, but resolution of closely appressed membranes by microscopy is difficult. Protease-protection assays and immune localization on intact apicoplasts have unequivocally demonstrated that PfoTPT resides in the outermost apicoplast membrane [12], but most other apicoplast

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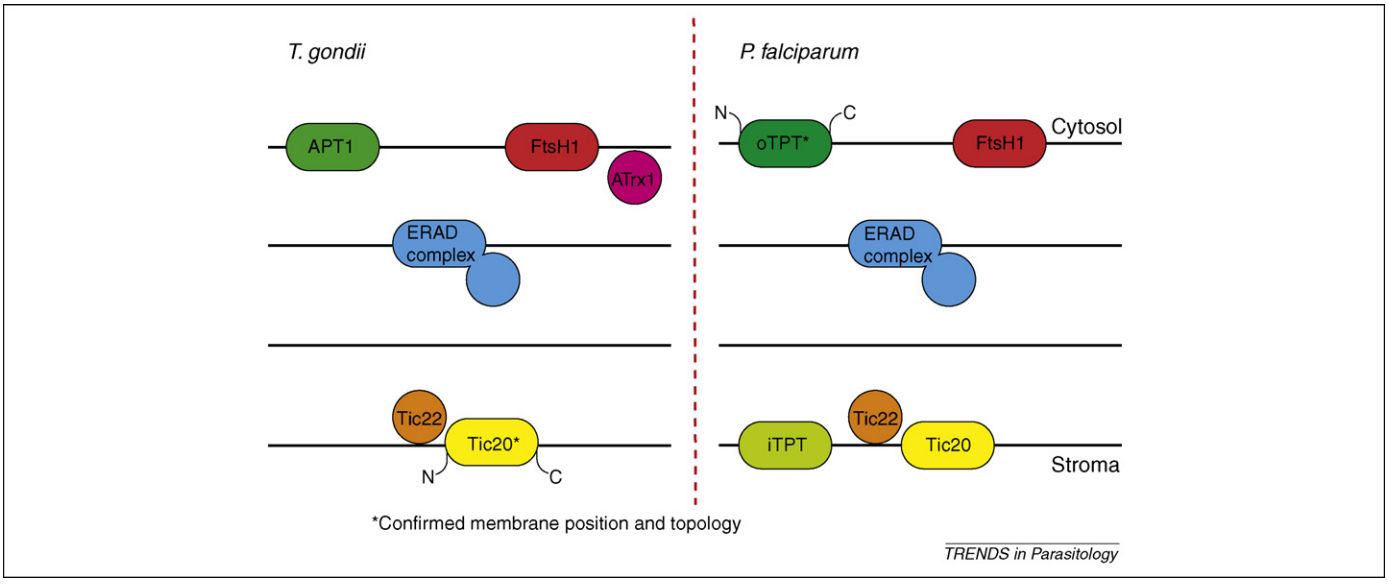

Figure 1. Known apicoplast membrane proteins in *T. gondii* and *P. falciparum*. The localization and topology of two apicoplast proteins, PfoTPT and TgTic20, have been confirmed experimentally. The innermost membrane localizations of PfiTPT, PfTic20, PfTic22 and TgTic22 are inferred from their evolutionary origins. The ERAD complex is postulated to reside in the periplastid membrane because of its secondary endosymbiotic origins [18]. The membrane proteins lacking a canonical bipartite leader (TgAPT1, TgFtsH1, TgATrX1 and PfFtsH1) are likely to be associated with the outermost membrane, analogous to PfoTPT, but their exact localization (perhaps even in multiple membranes [3,13,14]) remains to be determined.

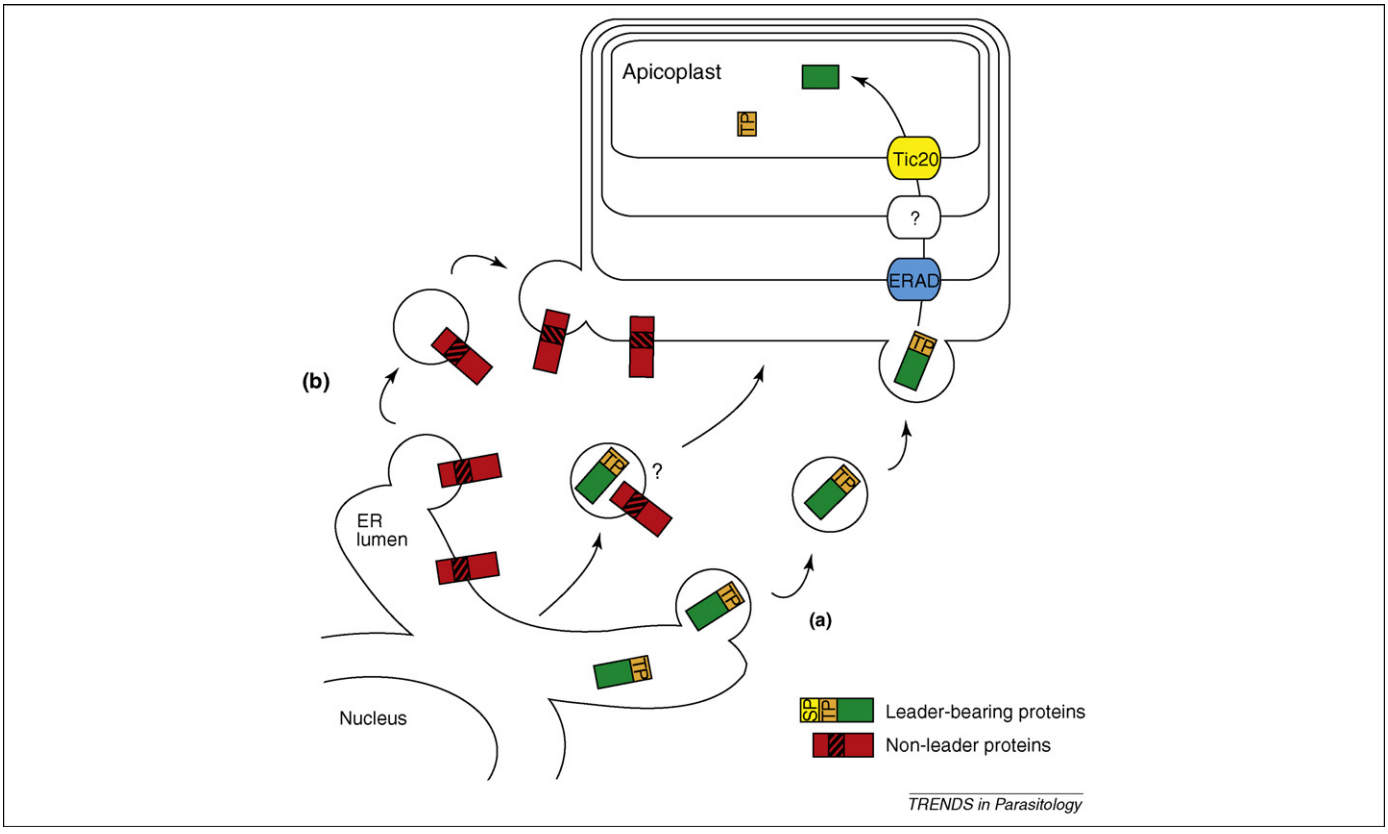

Figure 2. Two modes of apicoplast protein trafficking. Two targeting mechanisms are now known to mediate apicoplast protein trafficking: **(a)** a pathway involving the canonical N-terminal bipartite leader, consisting of a signal peptide, or SP (cleaved during co-translational insertion into the endomembrane system), and a transit peptide, or TP (cleaved within the stroma); or **(b)** a bipartite leader-independent pathway, probably involving proteins integral to, or associated with, the outermost membrane. How outer-membrane proteins are targeted to the apicoplast is currently unresolved, although a common feature seems to be the presence of a recessed signal anchor (diagonal lines), which might facilitate insertion into the endomembranes. Although proteins lacking bipartite leaders (b) have been observed in vesicle-like structures, bipartite-leader-bearing proteins (a) have not been observed in vesicles.

membrane proteins have not been localized so precisely (Figure 1). There is no precedent for the targeting of one protein into the multiple concentric membranes bounding mitochondria or chloroplasts.

The next frontier is to establish markers in each of the four apicoplast membranes in both *Plasmodium falciparum* and *T. gondii*. These keystones will enable more precise localization of novel apicoplast membrane proteins

and, it is hoped, will provide insight into how such protein targeting is mediated. The current apicoplast metabolic map is based primarily on genome mining to identify proteins bearing the distinctive bipartite leader [3]. DeRocher *et al.* [4] have pointed out the inherent limitation of this approach now that a new targeting route (at least for membrane proteins) has emerged. It is now paramount to dissect mechanisms of apicoplast membrane protein trafficking so that we can decipher how the organelle interacts with the surrounding cytosol. All four leaderless proteins (PfoTPT, TgFtsH1, TgAPT1 and ATrx1) in (or attached to) the outermost membrane possess a hydrophobic internal sequence that probably acts as an internal signal peptide, but we know nothing of how apicoplast membrane targeting and protein topology therein are mediated.

Electron-microscopy studies of ATrx1 [4] and TgAPT1 [13] reveal numerous vesicles in the vicinity of the apicoplast that are probably *en route* to the organelle. Because the apicoplast outermost membrane originated from the phagosomal membrane of the secondary host cell [8], vesicles bearing apicoplast membrane proteins (and/or soluble apicoplast proteins with bipartite leaders; Figure 2) are expected to be common and probably traffic proteins from the ER to the apicoplast [15,16]. Vesicles carrying ATrx1 are probably not coatomer protein II (COPII) vesicles because they are the wrong size [4,17]. Whether these vesicles are unique to trafficking apicoplast-destined proteins remains to be established. Puzzlingly, transport vesicles containing apicoplast stromal proteins have not yet been observed. Perhaps such traffic is more transient, or perhaps the reporter constructs utilized have rendered ephemeral membrane protein vesicular traffic visible.

Intriguingly, ATrx1 is apparently soluble or only peripherally associated with the apicoplast membrane [4]. This is in stark contrast to the integral membrane proteins PfoTPT, TgAPT1 and TgFtsH1 (Figure 1). DeRocher *et al.* [4] also observe multiple forms of ATrx1, which they attribute to either N-terminal processing or post-translational modifications. Further characterization, perhaps by N-terminal sequencing, should clarify the situation. Processing is also apparent for TgFtsH1 [4], but PfoTPT is clearly not processed [12]. Whether such protein processing correlates with the fidelity of apicoplast targeting or is protein-specific and related to function remains to be established.

First component of apicoplast protein-import machinery identified

The leader peptides of apicoplast-targeted proteins have been characterized extensively [9,10,16], but little is known about the machinery that recognizes these leaders and translocates the cargo across the organelle membranes (Figure 2). As outlined previously, the apicoplast outermost membrane is probably derived from the ER, and the observation of vesicles carrying apicoplast cargo corroborates this conclusion [4,15,16]. Import across the second outermost apicoplast (periplastid) membrane is hypothesized to be achieved via a duplicate ER-associated degradation (ERAD) complex [16,18]. Several ERAD components are apparent in the apicoplasts of *P. falciparum* and *T. gondii,* but none have been experimentally validated in transporting proteins [16,18].

The two innermost membranes of the apicoplast are probably homologous to the plant and red-algal chloroplast envelopes. Translocon of the outer chloroplast membrane (Toc) and translocon of the inner chloroplast membrane (Tic) complexes are postulated to facilitate protein transport across these two inner apicoplast membranes [19]. Thus far, no Toc components have been identified in parasites, but a second plastid-targeted ERAD component (Der1) is hypothesized to replace the function of a Toc [16]. Recently, van Dooren *et al.* [5] localized a Tic homologue (Tic20) to the innermost apicoplast membrane of *T. gondii* and concluded from knockdown experiments that TgTic20 is necessary for apicoplast protein import.

The *Toxoplasma* Tic20 homologue has a canonical bipartite leader that is probably processed by the putative SPP [5]. Tic20, which possesses four predicted transmembrane domains, was shown to be an integral membrane protein, and an elegant experiment using a split-green-fluorescent-protein (GFP) demonstrated that Tic20 resides in the innermost apicoplast membrane with its termini projecting into the apicoplast stroma [5]. To prove this, part of the GFP molecule was attached to the C terminus of Tic20 and the remainder of the GFP was targeted independently to the apicoplast stroma using the bipartite leader of a stromal protein. Neither GFP component can fluoresce alone, so fluorescence of a functional reconstituted fluorophore indicates that the two components reside in the same compartment. The split-GFP system has great potential for further characterizing apicoplast membrane and intermembrane space proteins.

Conditional mutants of *Toxoplasma* Tic20 failed to grow, and processing of proteins destined for the apicoplast stroma was repressed, suggesting a vital role for Tic20 in apicoplast import because bipartite leaders are removed only when proteins enter the apicoplast stroma [5]. Post-translational modifications of two apicoplast proteins were also affected when Tic20 was knocked down, complicating its role in apicoplast function [5]. The localization of Tic20 in the innermost apicoplast membrane and its demonstrated involvement in protein import confirm that apicoplasts inherited some of their protein-import machinery from the red-algal endosymbiont engulfed by an early ancestor of apicomplexan parasites and dinoflagellate algae [1].

Future perspectives

How many other apicoplast membrane proteins remain to be identified? Inner-membrane proteins (such as Tic20, and probably PfiTPT) bear canonical bipartite leader sequences and are readily identified at the primary sequence level. Proteins in the outer membrane are less straightforward to find, and we might need to identify them experimentally unless a characteristic sequence signature emerges in this vital group of membrane molecules.

The apicoplast inner-membrane transport protein Tic20 reminds us that the organelle is a highly modified plastid, homologous to those of plants and algae [19]. Other Tic components are evident in the genomes of parasites

(Figure 1), but the apparent lack of Toc components, the possible recruitment of the ERAD complex for apicoplast protein import and the novel targeting mechanism for outer-membrane proteins serve to caution us against basing our models solely on evolutionary comparisons between apicoplasts and their photosynthetic relatives. It is also noteworthy that extrapolating studies from sister organisms might not always be a relevant exercise, as demonstrated by the apparent lack of an ATrx1 homologue in *P. falciparum* and the presence of just one sugar-phosphate transporter (TgAPT1) in the apicoplast of *T. gondii* [4,13].

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Research Focus

Monkey malaria kills four humans

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Four human deaths caused by *Plasmodium knowlesi*, a simian malaria species, are stimulating a surge of public health interest and clinical vigilance in vulnerable areas of Southeast Asia. We, and other colleagues, emphasize that these cases, identified in Malaysia, are a clear warning that health facilities and clinicians must rethink the diagnosis and treatment of malaria cases presumed to be caused by a less virulent human malaria species, *Plasmodium malariae*.

Cause of death revealed

A paper by Cox-Singh *et al.*, entitled '*Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening' [1], represents a telling retrospective study of blood-smear samples from four patients who died of malaria and addresses the fact that *P. knowlesi* transmission is more widespread than imagined previously. The four patients (who were from 39 to 69 years old) were being treated for human *Plasmodium malariae* infections, in accordance with their original blood-smear diagnoses. These patients had various common clinical features, including high fever, abdominal pain, acute renal impairment, jaundice and thrombocytopenia, which are atypical for *P. malariae* infections. Indeed, PCR and sequencing revealed that *P. knowlesi* malaria was probably the actual cause of death.

Plasmodium knowlesi infections in humans

Plasmodium knowlesi, initially identified in 1931 in a *Macaca fascicularis* monkey originating in Singapore, was shown in 1932 to be capable of infecting humans

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